UNIVERSITY OF MINNESOTA

SUMMARY OF PROGRESS IN THE PROJECT ENVIRONMENTAL MICROBIOLOGY AS RELATED TO PLANETARY QUARANTINE

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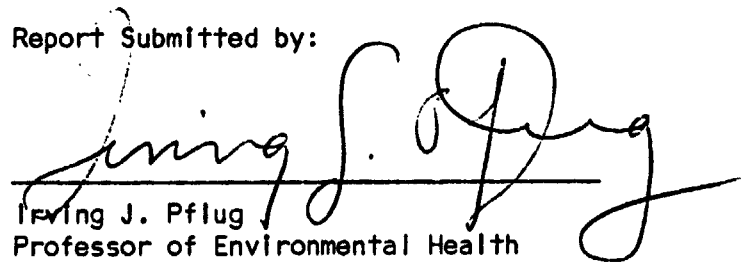

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SUMMARY

During the period covered by the current report, the Environmental Sterilization Laboratory, Space Science Center, University of Minnesota has continued experimental work related to dry heat resistance of microorganisms. One phase of this research has been concerned with the viability and dry heat resistance of indigenious microflora associated with small soil particles. Another series of investigations has been directed toward special analyses of Bacillus subtilis spore crops produced and stored in the laboratory.

For the studies of the microscopic sized (74-88 µm) soil particles and the dry heat effects on associated microorganisms, we have developed an Aluminum Boat-TDT Cup-TSA solid media system. This technique appears to facilitate the assay procedure for particle viability testing. Modification of the TSA media by addition of triphenyltetrazolium chloride improved visible detection of microbial growth. Details of the procedure are described in this report.

The data included in the present report were obtained using analyses of individual soil particles. Detailed particle viability profiles for dry heat effects were determined for Kennedy Space Center soil. At 110°C at least some particles retained viability through a heating period of between 8 and 16 hours. Single particles heated at 125°C for 80 minutes or longer did not show evidence of viability under test conditions.

Preliminary aerobic, mesophilic plate counts of the 74-88 µm soil fraction yielded mean values of 16.2 organisms per dark particle and 2.6 organisms per light particle. Heat treatment of particles in a dry atmosphere did not appear to increase the rate of inactivation for in situ soil particle microflora.

Investigation relevant to the storage of Bacillus subtilis spores is being continued. This research is attempting to analyze the effect of storage temperatures, cleaning procedures, storage time and suspending medium on the stability and dry heat resistance of B. subtilis var niger spores. The work is a long term study which will run over a three year period with semi-annual analyses of the spore crops.

DRY HEAT EFFECTS ON VIABILITY OF CAPE KENNEDY SOIL PARTICLES

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INTRODUCTION

Fallout of small soil particulates from the air of assembly areas and transfer of particles to components by assembly workers may be sources of microbial contamination for space probe units. The resistance to dry heat sterilization of indigenous soil microflora associated with individual soil particles is, therefore, of interest to the planetary quarantine research program.

In the past, earlier investigations have dealt with dry heat resistance characteristics of cleaned spore suspensions obtained from laboratory cultures of selected spore forming bacteria. These studies have provided extensive data on dry heat destruction rates for unprotected spores under a variety of conditions. Results accumulated from thermal resistance analyses of spores in various laboratories have formed the basis for assessment of heat treatment requirements to be used on spacecraft.

Currently, supplementary information concerning the viability of individual soil particles, and the associated microorganisms, when subjected to dry heat treatment cycles appears desirable. This is especially important because previous studies have suggested that the indigenous flora of natural soil environments may, in some instances, demonstrate a greater resistance to heat treatment than spores of laboratory cultured isolates. Data dealing with the rates of soil particle inactivation and loss of viability can be of assistance to the designers of space probe sterilization processes. The compilation of information on dry heat effects on microbial assemblages associated with various soil particulates should also be useful in the selection of sterilization cycles.

OBJECTIVES

The objective of this project is to determine the dry heat resistance patterns for typical sized particles of Cape Kennedy soil.

Specific objectives of these experiments were as follows: (1) to further develop and improve the system for testing soil particle viability after heat treatment, (2) to determine dry heat resistance characteristics of Cape Kennedy soil (WAKMF 74-88 μm) particles at 110°C and 125°C, (3) to determine effects of conditioning and heating in low vs relatively higher moisture levels on soil particle viability, (4) to estimate the microbial concentrations associated with the 74-88 μm soil particles using plate counts.

MATERIALS AND METHODS

Soil Particle Viability Studies

This series of investigations was concerned with dry heat resistance of *in situ* microorganisms associated with soil particles. In the pursuit of the research, certain modifications in culture media, techniques and equipment were necessary. The specific procedures which were developed for the soil particle viability studies are outlined in sections that follow.

Soil Samples. The Cape Kennedy soil used in the present investigations was designated by the code, WAKMA. This sample was received by our laboratory on June 18, 1973 in the form of a five-pound bulk shipment of dry soil. Soon after delivery, the soil sample was separated into a series of particle size ranges by shaking for 30 minutes on a Ro-Tap machine equipped with standard ASMT-NBS sieves. Prior to use, the sieves were thoroughly washed with a detergent solution, rinsed with distilled water, ethanol rinsed and dried.

Soil fractions were transferred to clean glass jars and stored until used. Of the soil fractions collected, only the intermediate size particles in the 74-88 μm range were used for the investigations described in this report.

Particle Selection and Transfer. Individual 74-88 μm particles were separated and used in the heat treatment studies. For particle selection, approximately 0.5 g of the soil fraction was transferred to a clean microscope slide using a sterile stainless steel spatula. A three-jawed micro-extension clamp, attached to a ring stand served as a holder to position the slide over the microscope stage.

Particles were spread out in a mono-layer on the slide and separated

with the aid of a stereoscopic microscope (magnification range 20-60X) and a sterile, fine, chromel wire probe. Single particles were transferred by micro-manipulation and placed in individual, sterile stainless steel TDT cups (7/16 in. dia. X 3/8 in. deep) arranged in orderly rows in a petri dish.

Electrostatic charge effects on the particles were sufficient to cause them to cling to the wire probe. Therefore, with careful handling, transfers of particles could be made directly without need for additional equipment. Except for special plate count studies of light vs dark particles, all other particles were transferred non-discriminately, i.e. without any concious effort to select them on the basis of structure, crystalline appearance, color, etc. All particle transfer work was done in a laminar cross flow clean bench.

Heat Treatment Units. After all particles of a set to be tested had been placed in TDT cups, sterile forceps were used to transfer the cups to aluminum boats used for heating. These units were 4 in. dia. X 3/8 in. thick, hexagonal shaped, aluminum plates machined in our laboratories. Each boat was drilled to contain 37 equally spaced wells (7/16 dia. X 1/4 in. deep) to accomodate the TDT cups.

Following the placement of all cups into boat wells, each boat was covered with a 4 in. petri dish top as a precautionary measure against any possible fallout contamination during and after the heating process. Special shoulder sections machined into each of the six corners of the boats held the cover in position. The covered boats were then placed inside a large (6 in.) petri dish which served as a carrier for transport of units from one laboratory area to another.

All boats were heated on 110°C or 125°C hotplates for selected times, then cooled three to five minutes on a cold plate (circulating water). After cooling, boats were returned to the large petri dish carriers. Further processing of all samples was carried out in a laminar downflow clean room facility using strict aseptic procedures.

Media Preparation and Addition to Cups. For our investigations, a Trypticase Soy Agar--Triphenyltetrazolium Chloride (TSA-TTC) medium was used to determine particle viability in TDT cups. The 2,3,5 Triphenyltetrazolium Chloride* is a redox indicator that is soluble

*Fisher Chemical Co. Cat. # T-413.

in water and colorless in the oxidized form. When the dye is reduced, as by the metabolic activity of living microorganisms, a red formazan precipitate forms at the site of microbial growth. The precipitate is insoluble in water and remains localized at the area of microbial colony development.

As used in our studies, a 1.0% aqueous solution of the TTC dye was prepared. This was passed through a sterile 0.45 µm MF filter unit, collected in a series of sterile, 15 ml capacity vials and stored at 4°C. Vials were individually wrapped in aluminum foil to exclude light. The TSA media was dispensed in 20 ml or 30 ml quantities in test tubes, sterilized by autoclaving and stored. To prepare the TSA-TTC media, the TSA was first melted and cooled slightly. Then the redox indicator was added aseptically in amounts to provide a concentration of 1.0 ml of 1% TTC per 100 ml of TSA media.

All media mixing and pipetting was done in the clean room. When the media was ready for delivery to TDT cups, the large petri carrier top was removed and the smaller cover lifted to allow access to the cups. As a precaution against contamination, sterile rubber gloves were used on hands that moved near cups during pipetting. Approximately five drops of melted TSA-TTC media was delivered to each TDT cup with a 10 ml pipette. A separate tube of media and a separate sterile pipette for one time use per boat was an additional measure instituted to avoid chance contamination.

Incubation of Cups. After media additions to all cups was completed, the 4 in. petri top was replaced. Ten ml of sterile, distilled water was added to the bottom of the 6 in. petri dish carrier, the top replaced and the unit sealed at the joint with masking tape. The water addition and sealing was necessary to prevent drying of agar in the cups over prolonged incubation periods used. All boats were incubated at 32°C for two weeks.

Recording Data. At the end of the incuabtion period, cups in each boat were examined individually under a stereoscopic microscope. The formation of a red colored formazan precipitate around the particle or other visible evidence of colony development was interpreted as a positive response and this was recorded as a viable particle. By determining the number of particles remaining viable after heating times, the results could be expressed as a proportion positive value. These

data were recorded in fractional and decimal terms. The ratios of the fraction of particles with viable organisms were also plotted on a graph as a function of heating time.

Dry Box vs Clean Room Systems. Most of the experimental studies concerned with the dry heat resistance of soil particles were done in the laminar downflow clean room. The air system of the room was controlled at 22 C and 50%RH (approximately 13,000 ppm moisture).

In addition to clean room work, a series of several experiments were also run to compare the viability of soil particles heated in a dry atmosphere with th viability of similar particles treated under relatively more moist conditions of the clean room environment. The dry atmosphere tests were run in a glove box unit, previously modified to provide a dry gas environment. Dry nitrogen gas, at a constant controlled flow, passed through the unit and a humidity control system was included in the gas train. A moisture monitor provided a continuous record of the glove box humidity. Hotplates operating at 110°C and 125°C were installed in the dry box. Continuous temperature records were obtained with thermocouple probes and a Hewlett-Packard multi-channel data acquisition system. The dry box atmosphere was controlled at approximately 27°C and 0.28%RH (100 ppm moisture). Additional details of the dry atmosphere system have been described previously (see University of Minnesota, School of Public Health NASA Report, #9, Appendix A).

Soil particles studied in the Clean Room-Dry Box series were also placed in individual TDT cups. These cups were transferred to the aluminum boats set in large petri dishes. The units were then placed in the clean room or dry box atmosphere for a period of 18 hours conditioning with the 6 in. petri carrier covers removed but with the smaller, 4 in. covers in place. Both the clean room and dry box sets of particles were heated on the same day. Following heat treatment, the dry box units were processed by procedures described in the other sections above.

Estimates of Particle Microbial Load

These preliminary experiments were done to obtain plate count data on the number of aerobic, mesophilic microorganisms associated with light and dark soil particles. Also of interest was the effect of selected heat treatments on the microbial counts and particle viability.

Cape Kennedy soil, Code WAKMF (74-88 μm), was the fraction used in all experiments. Three sets of 74 individual soil particles per set were

studied. Each set was comprised of 37 individual light colored and 37 dark colored particles. All particles were selected by micro-manipulation with the aid of a stereoscopic microscope and each particle was placed in a separate TDT cup for the analysis. All processing of cups was done in the clean room.

The three sets of particles were treated as follows:

Set A. No heat treatment. Each TDT cup, with the individual particle, was placed in a 125 ml Erlenmeyer flask with 25 ml phosphate buffer and insonated for two minutes. Then 25 ml double strength Trypticase Soy Agar (TSA) medium was added, the flask contents mixed well, and plated in two petri dishes.

Set B. Soil particles, each in individual cups, were insonated in flasks with 25 ml of buffer. After insonation, each flask was heated in a water bath at 80°C for 20 minutes, cooled 30 seconds in an ice bath, and then plated as in Set A.

Set C. Individual particles in TDT cups were placed in aluminum boats. Boats were heated on the clean room hotplate at 110°C for one hour, cooled, and then each cup was sonicated in a flask with 25 ml buffer. After insonation, flask contents were plated as described for Set A.

All plates were incubated at 32°C for six days after which colony counts were done. The longer incubation time was used because a substantial number of colonies on some plates were slow growing actinomycetes. Data were reported in terms of microbial colonies per soil particle.

RESULTS AND DISCUSSION

Earlier work with WAJJ soil particles studied in our laboratory had been done with small capacity heating units (boats with wells for only five cups). Those investigations employed a liquid medium (Trypticase Soy Broth supplemented with 0.005% alanine) to detect survival of microorganisms on heated particles. The use of liquid media to test particle viability required considerable additional handling of cups and tubes after the particles had been heated. This was a disadvantage when processing larger numbers of particles. At times, the occurrence of only slight growth was also somewhat difficult to detect in the liquid culture tubes.

Studies to Improve Viability Detection

In an effort to improve the procedures for particle viability analyses,

three modifications have been made recently. These were as follows: (1) use of 37-well aluminum boats for heating, (2) addition of solid agar media directly to the TDT cups and (3) the use of TTC redox indicator in the media. All of these changes in methodology were found to facilitate the testing of soil particulates and aided in the detection of viable particles.

Employment of boats with 37 wells provided increased capacity and reduced handling for the test of particles. By employing these units, at least 74 particles or more were tested for every heating time interval studied. It was found that the use of aluminum boats facilitated transfer and heating of the particles in a test series.

The use of agar media pipetted directly into TDT cups after particle heating was a distinct advantage over the use of liquid, tubed media. Firstly, direct pipetting of agar media to cups precluded the previous need for extra handling of cups and large numbers of liquid media tubes after the heating process. Secondly, the particles in cups with agar could be inspected directly under the microscope for any minute evidence of growth. Lastly, the use of solid media, and the consequent reduction in handling, also reduced the exposure of heated samples to any possible extraneous contamination from added manipulation.

Upon observing the nature and extent of microbial growth from the soil particles in agar, it seemed desirable to seek a modification of the TSA media to promote easier recognition of the minute amounts of growth found in close proximity to the soil particle. Therefore, in an attempt to improve the media sensitivity for detecting particle viability, a series of experiments using TSA media with TTC additions were run.

The data listed in Table I show the results of preliminary trials with soil particles in TDT cups. This series was run to ascertain the feasibility of using the redox indicator in media added to cups.

Three different sets of 15 to 17 cups, each with a single soil particle, were treated as shown in the table. The addition of TSA-TTC media to the unheated particles resulted in easier detection of the viable particles which comprised about 64% of the total tested in this group. Of the 17 soil particles heated at 110°C for one hour, four retained viable organisms as indicated by growth and indicator reduction. Evidence of increases in numbers of viable particles was observed over the two week period. No change in the indicator was evident around any of the soil particles treated

Table 1

Results from Trials with TSA-TTC Media
and 74-88 μm Soil Particles ** in Cups

Type of Dry Heat Treatment	Time of Readings					
	3 days		7 days		2 weeks	
	pos.*	neg.	pos.	neg.	pos.	neg.
None	9	8	11	6	11	6
110°C--1 hour	0	17	1	16	4	13
180°C--3 hours	0	15	0	13	0	15

* Pos. refers to number of particles with microbial growth
** Random particles

Table 2

Results with Soil Microorganisms Initially Isolated From
Particles Plated on TSA with Subsequent Transfer to TSA-TTC Media

Colony Size on TSA	No. of Isolates from TSA	No. Showing Growth and Red PPT.	Percent
Readily detectable	30	30	100
Doubtful colonies	5	0	0

at 180°C for three hours. This result suggested that when the microflora of the particles were rendered nonviable, no spontaneous reduction of the indicator occurred under test conditions.

In all cases, the TSA-TTC medium gave a readily detectable, bright red precipitate in the agar around each soil particle in cups with evidence of growth. These results indicated that TTC indicator would be useful as an aid to easier recognition of particle viability.

Following the initial encouraging results with TSA-TTC media, an experiment was run to test the ability of various natural soil microflora isolates to grow readily on this medium. It was of special interest to know whether any inhibiting effects might result from the use of the TTC. For this study, Cape Kennedy soil particles (74-88 μm) were initially sonicated in buffer and plated in petri dishes with TSA media. Following colony development, a series of about two dozen plates were chosen specifically to obtain a variety of colony types as indicated by appearance. This selection was made to obtain as wide a variety of colonies as possible, including actinomycetes as well as bacteria. From these plates, 30 well defined, different, large, and small colonies were transferred to TSA-TTC agar plates. Five doubtful areas that might have been inclusions in the agar or small pin point colonies were also transferred to the TTC plates. The plates were read after incubation at 32°C for four days.

Results of the response from natural soil particle isolates on TTC media are listed in Table 2. All of the 30 isolates transferred from TSA grew and developed a readily detectable red precipitate on the TTC media. None of the five doubtful colony areas showed any evidence of growth on the TTC agar. There is a possibility that this last group of five transfers may have been unable to develop on TTC media. However, since all the other 30 diverse colonies did grow well, it is more likely that these pinpoint areas may have been media artifacts.

Also of interest in the trial work with TTC was the question of its effect on aerobic spore formers. For this reason, an experiment was done to determine if the addition of the indicator to TSA media would cause any inhibitions of growth or affect colony counts of Bacillus subtilis var. niger.

For the study, a test spore suspension of B. subtilis (AAHK) was used. The original suspension was diluted with buffer to obtain a concentration

of approximately 150 spores per ml. Quantities of 1.0 ml and 0.1 ml were plated in a series of 60 plates for each dilution. Thirty plates of the 1.0 ml and thirty of the 0.1 ml volumes were poured with TSA media. The remaining 60 plates were poured with TSA-TTC. Plate counts were made after incubation at 32°C for 48 hours.

Table 3 lists the results obtained from comparison plate counts of B. subtilis on TSA and TSA-TTC media. These data indicate that this spore former developed about the same mean number of colonies on both media. The mean values obtained for colony counts suggest that sporulation and colony development occurred without any inhibition by the indicator dye.

The results of the studies on TTC additions to the media indicate that TSA-TTC will generally support the growth of the same variety of soil associated species that develops on TSA media. The addition of the redox indicator did not appear to be inhibitory to isolates tested. However, the formation of the red precipitate made the smaller colonies more easily detectable than they would be on TSA alone. This is a beneficial factor in reading results of particle response to treatment in TDT cups.

Plate Counts of Particles

Plate count analyses of the soil fraction were initiated to obtain information on the mean microbial loads associated with the individual particles. Both light and dark particles were analyzed. Plate counts were made of aerobic, mesophilic microorganisms and of heat resistant microflora indigenous to WAKMF soil particles. Results obtained from this series of experiments are summarized in Table 4.

These plate count data indicate that dark soil particles carried a higher number of microorganisms than the light particles. The mean count per dark particle was six times greater than that for light ones (i.e. 16.2 organisms/particle as compared to 2.6 organisms/particle). It is interesting to note that all of the unheated dark particles tested were viable; however, only 60% of the untreated light particles produced colonies on plates.

Among the dark particles tested, a much higher proportion remained viable after wet heat treatment at 80°C for 20 min. than was observed for light colored particles--about 92% as compared to 32%. The mean count of the dark particles was ten times greater than the count for light particles treated at 80°C for the same time interval.

Table 3

Plate Counts of Bacillus subtilis var. niger
on TSA and TSA-TTC Media (32°C-48 hr.)

Amount of Spore Dilution Plated (ml)	Number of Colonies Per Plate			
	TSA medium		TSA-TTC medium	
	mean	range	mean	range
1.0	127.6	108-160	139.1	113-203
0.1	12.9	3-22	13.1	4-22

Table 4

Heat Treatment Effects on Viability of Light and Dark Particles and Plate Count Estimates of Microbial Load Associated with WAKMF (71-88 μm) Fraction Cape Kennedy Soil (Data from Exps. OR3289A, OR3290A, and OR3291A)

Treatment	Proportion Positive **				Plate Count Mean org/particle	
	Light		Dark		Light	Dark
	fraction	decimal	fraction	decimal		
None	22/37	(0.595)	37/37	(1.00)	2.6 (0-18)*	16.2 (1-46)
Wet Heat (20 min at 80°C)	12/37	(0.324)	34/37	(0.919)	0.5 (0-4)	5.1 (0-35)
Dry Heat (60 min at 110°C)	1/37	(0.027)	27/37	(0.729)	0.03 (0-1)	1.9 (0-10)

* Indicates range of counts per particle
** Refers to fraction of particles with viable microorganisms.

Similar to the results at 80°C, highest counts of microorganisms able to survive at 110°C dry heat for one hour also appeared to be associated with dark soil particles. Approximately 73% of the dark particles produced growth after the dry heat treatment; however, only 2% (one particle in 37) of the light particles demonstrated viability after heating at 110°C for one hour. After treatment at 110°C, the mean count for dark particles was reduced to 2.9 organisms/particle, while for the light fraction the mean count was only about one organism/35 particles.

Although both light and dark particles demonstrated a reduction in numbers remaining viable after heat treatments, the effect was more pronounced on the light particles. On the basis of these limited observations, the dark particles appear to harbor a greater number of microorganisms and also seem to be more resistant to heat treatment.

Heat Effects on Particle Viability

A series of ten experiments was completed to study the effects on particle viability of heating at 110°C for selected times. The results of these studies are presented in Table 5 and Figure 1. These experiments covered heating times that ranged from one to 24 hours. Data compiled from this investigation are based on the transfer, heating, and analysis of 740 individual soil particles.

Table 5 lists the fraction of particles that retained viable microorganisms for each heating time interval. The unheated set of particles tested yielded 60 viable particles out of 74 which represents a viability of about 81%. After one hour of heating, the proportion of particles demonstrating growth had been reduced to approximately 39%. Nearly three percent of the particles heated for eight hours still remained viable. All of the particles heated for 16 hours and 24 hours failed to show evidence of growth under the test conditions.

The trend of particle inactivation with heating time is illustrated in Figure 1. From the results of these experiments, it appears that the reduction in number of viable particles with time is relatively uniform for the series tested at 110°C. For purposes of comparison with other data, the rate of particle inactivation is reflected in the slope of the graph.

Two series of soil particles were run to obtain information on particle inactivation by dry heat at 125°C. The first experimental series

Table 5

Soil Particle Viability After Dry Heat Treatment on Clean Room Hotplate at 110°C

WAKMF Soil (74-88 μm). Proportion Positive Data. *

Experiment Number	Heating Time (hours)	Proportion Positive	
		Fraction	Decimal
OR3296A	0	60/74	0.811
OR3296B	1	29/74	0.392
OR3298A	2	16/74	0.216
OR3311A	3	10/74	0.135
OR3298B	4	6/74	0.081
OR3311B	5	7/74	0.095
OR3311C	7	2/74	0.027
OR3303A	8	2/74	0.027
OR3303B	16	0/74	0.000
OR3305A	24	0/74	0.000

* Refers to fraction of particles with viable microorganisms.

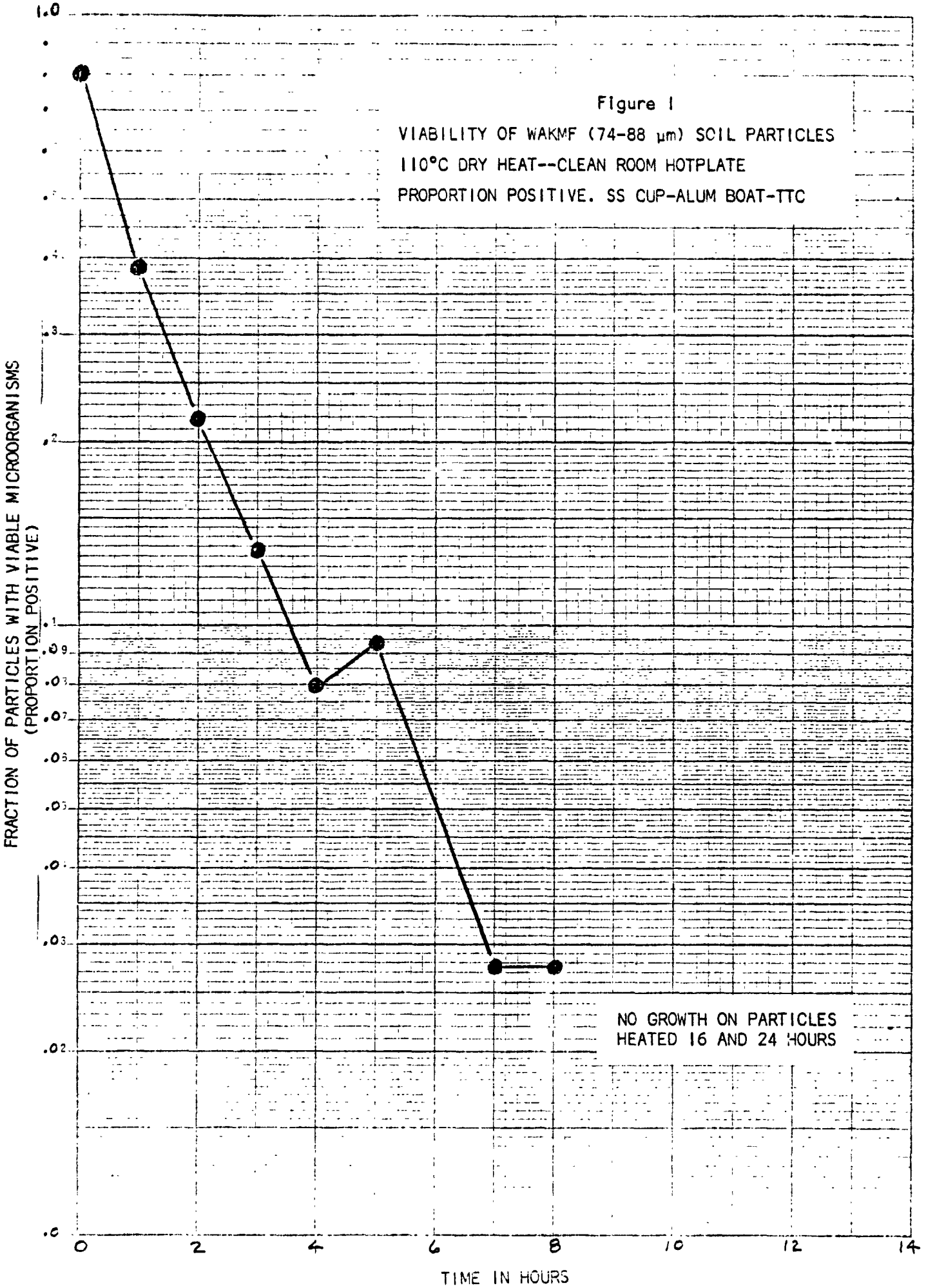
Figure 1
VIABILITY OF WAKMF (74-88 µm) SOIL PARTICLES
110°C DRY HEAT--CLEAN ROOM HOTPLATE
PROPORTION POSITIVE. SS CUP-ALUM BOAT-TTC
FRACTION OF PARTICLES WITH VIABLE MICROORGANISMS
(PROPORTION POSITIVE)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.0
0
2
4
6
8
10
12
14
TIME IN HOURS
NO GROWTH ON PARTICLES
HEATED 16 AND 24 HOURS

(OR3310 to OR3319) included a total of 592 individual particle analyses and a heating time interval range of 30 min. to 360 min. Proportion positive data from this investigation are listed in Table 6 and plotted on the graph of Figure 2.

Inspection of the data in Table 6 shows that approximately 76% of the unheated particles tested showed evidence of viability. Following 60 min. of heating at 125°C, viability was detected in only one particle out of 74 tested. For the heating times from 120 min. to 360 min., all of the particles analyzed were negative for microbial growth under the test conditions used. The fact that the last five sets of 74 particles each were negative is interesting for two reasons. First, the data suggest that for this series of 74-88 μm particles the time for inactivation at 125°C appears to lie somewhere between 60 min. and 120 min. And, second, the fact that five successive sets of cups (representing 10 separate boats and 370 particles) were all negative indicates that our techniques to avoid extraneous contamination were satisfactory.

A second experimental series at 125°C was run with shorter heating times. This was done to obtain a more detailed particle inactivation profile than the first series provided. Table 7 lists the proportion positive data representing 518 particles tested in experiments OR3331 through OR3338 of this study. The corresponding graph of proportion positive values for heating times is shown in Figure 3. These data tend to confirm the results of the first experimental series in that some particles again retained viability through heating times of 60 min., but none were positive when tested after 80 min. of heat treatment.

Data from both 125°C experimental series (OR3310 through OR3338) have been combined in Table 8 which, therefore, represents the results accumulated from processing of 1,110 individual soil particles. A refined particle inactivation profile was graphed from these values and is shown in Figure 4. Because the basis for this graph is a much larger sampling of soil particles than previously tested, it would appear that these results may be somewhat more representative of the WAKMF soil particles response to dry heat at 125°C.

Dry Box vs Clean Room Studies

Several series of experiments were done to determine the effects of conditioning and heating soil particles in a dry environment as contrasted

Table 6

Soil Particle Viability After Dry Heat Treatment on Clean Room Hotplate at 125°C

WAKMF Soil (74-88 µm). Proportion Positive Data. *

Experiment Number	Heating Time (minutes)	Proportion Positive	
		Fraction	Decimal
OR3310A	0	56/74	0.757
OR3310B	30	13/74	0.176
OR3313A	60	1/74	0.014
OR3313B	120	0/74	0.000
OR3317A	180	0/74	0.000
OR3317B	240	0/74	0.000
OR3319A	300	0/74	0.000
OR3319B	360	0/74	0.000

* Refers to fraction of particles with viable microorganisms.

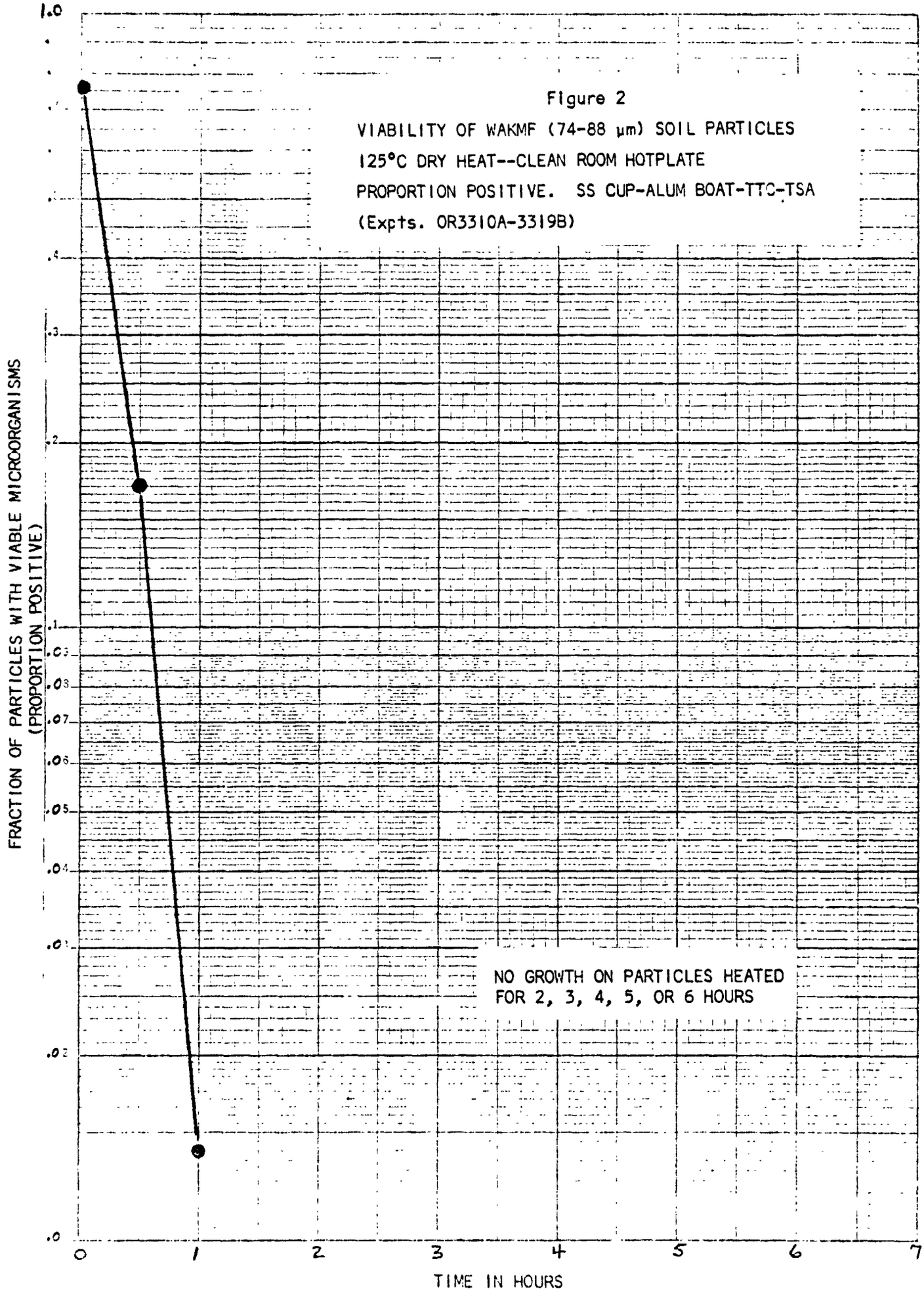
Figure 2
VIABILITY OF WAKMF (74-88 µm) SOIL PARTICLES
125°C DRY HEAT--CLEAN ROOM HOTPLATE
PROPORTION POSITIVE. SS CUP-ALUM BOAT-TTC-TSA
(Expts. OR3310A-3319B)
FRACTION OF PARTICLES WITH VIABLE MICROORGANISMS
(PROPORTION POSITIVE)
1.0
.4
.3
.2
.1
.03
.08
.07
.06
.05
.04
.01
.02
.0
NO GROWTH ON PARTICLES HEATED
FOR 2, 3, 4, 5, OR 6 HOURS
0
1
2
3
4
5
6
7
TIME IN HOURS

Table 7

Soil Particle Viability After Dry Heat Treatment on Clean Room Hotplate at 125°C.

WAKMF Soil (74-88 µm). Proportion Positive Data. *

Experiment Number	Heating Time (minutes)	Proportion Positive	
		Fraction	Decimal
OR3331A	0	65/74	0.878
OR3331B	10	28/74	0.378
OR3331C	20	13/74	0.176
OR3333A	30	6/74	0.081
OR3333B	40	5/74	0.068
OR3338A	60	3/74	0.040
OR3338B	80	0/74	0.000

* Refers to fraction of particles with viable microorganisms.

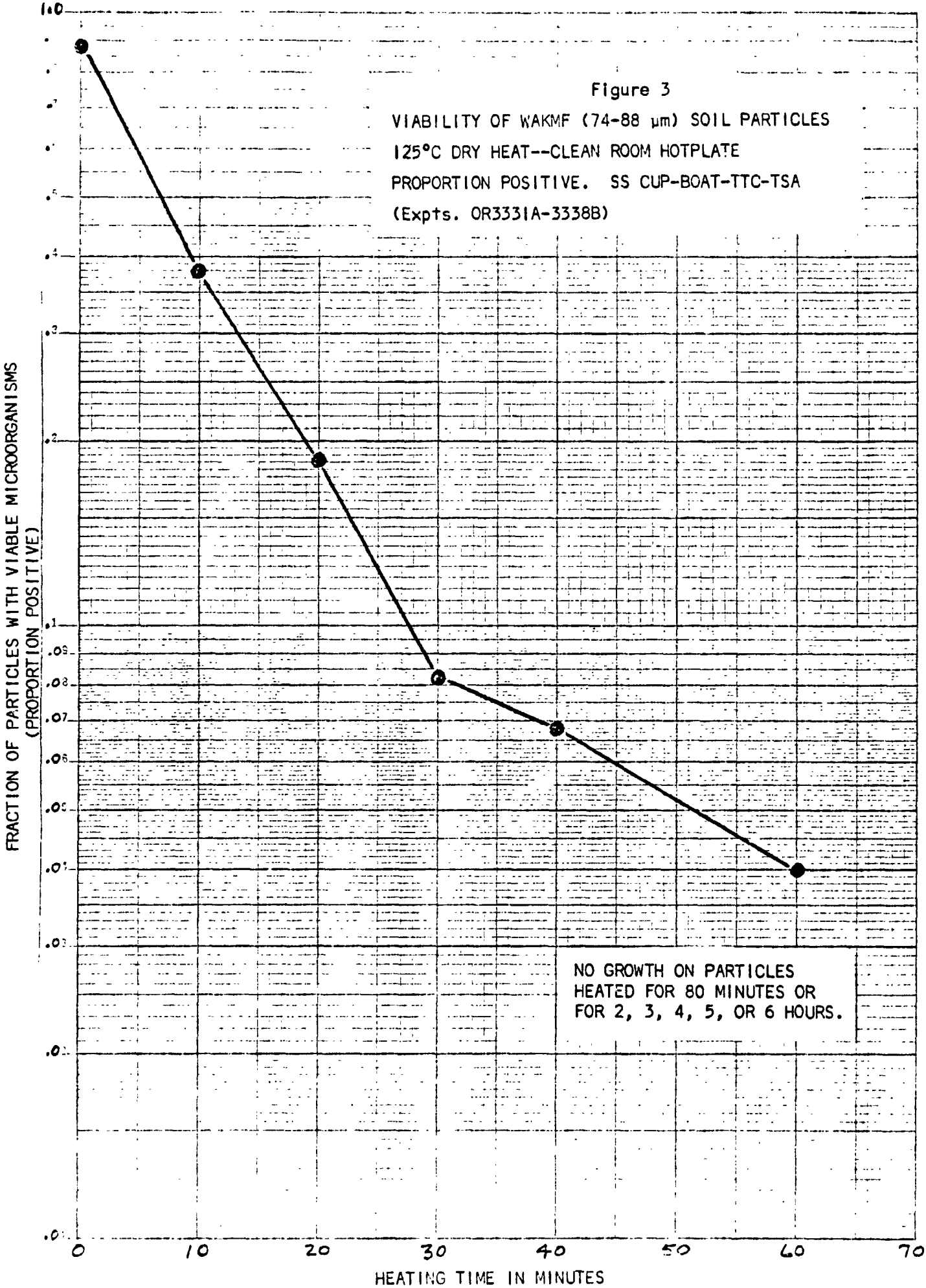
Figure 3
VIABILITY OF WAKMF (74-88 µm) SOIL PARTICLES
125°C DRY HEAT--CLEAN ROOM HOTPLATE
PROPORTION POSITIVE. SS CUP-BOAT-TTC-TSA
(Expts. OR3331A-3338B)
FRACTION OF PARTICLES WITH VIABLE MICROORGANISMS
(PROPORTION POSITIVE)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
10
20
30
40
50
60
70
HEATING TIME IN MINUTES
NO GROWTH ON PARTICLES
HEATED FOR 80 MINUTES OR
FOR 2, 3, 4, 5, OR 6 HOURS.

Table 8

Soil Particle Viability After Dry Heat Treatment on Clean Room Hotplate at 125°C.

WAKMF Soil (74-88 μm). Proportion Positive Data. *

(Composite Summary of All Experiments)

Experiment Number	Heating Time (minutes)	Proportion Positive	
		Fraction	Decimal
OR3-310A,331A	0	121/148	0.818
OR3331B	10	28/74	0.378
OR3331C	20	13/74	0.176
OR3-310B, 333A	30	19/148	0.128
OR3333B	40	5/74	0.068
OR3-313A, 338A	60	4/148	0.027
OR3338B	80	0/74	0.000
OR3313B	120	0/74	0.000
OR3317A	180	0/74	0.000
OR3317B	240	0/74	0.000
OR3319A	300	0/74	0.000
OR3319B	360	0/74	0.000

* Refers to fraction of particles with viable microorganisms.

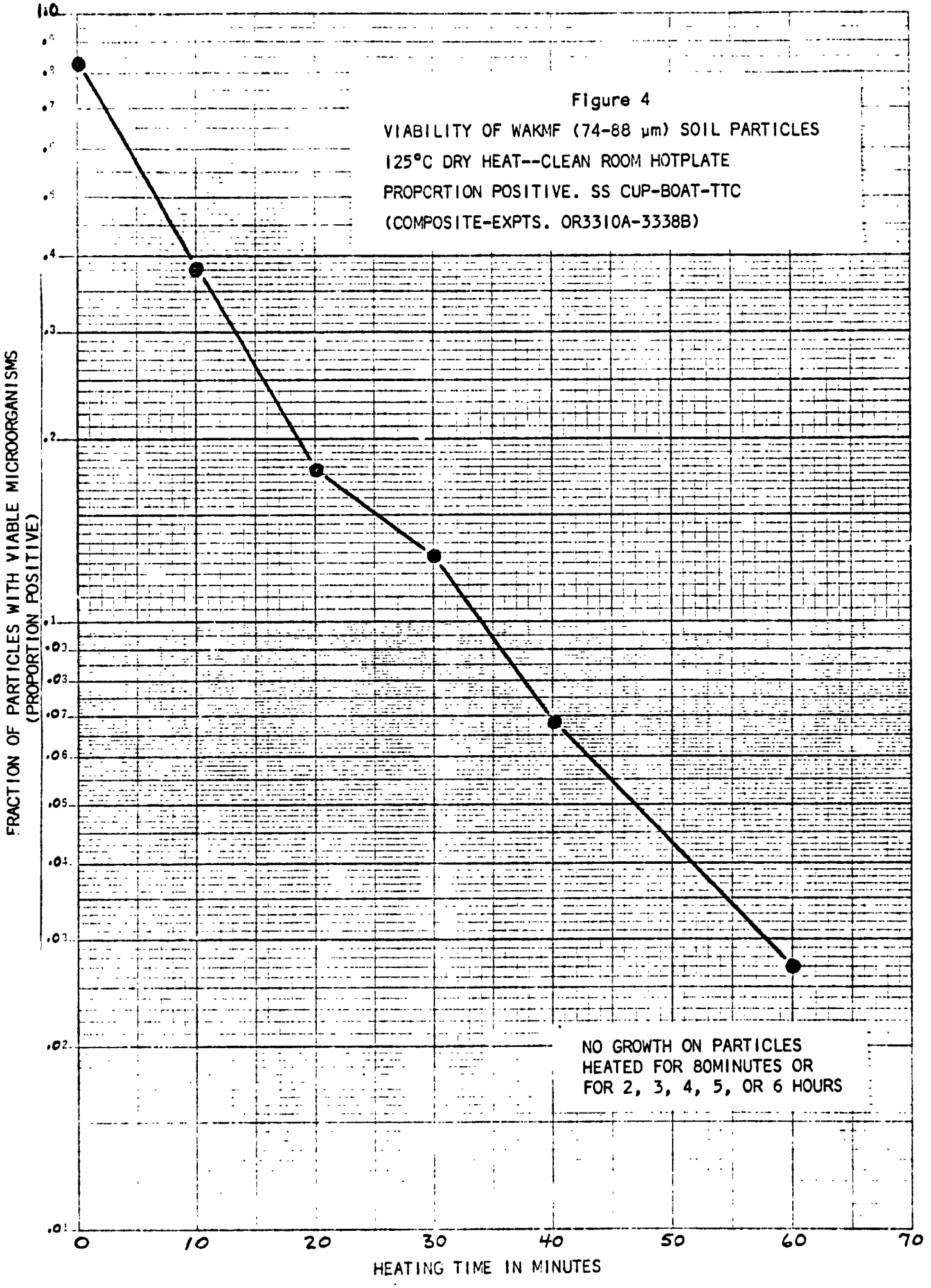
Figure 4
VIABILITY OF WAKMF (74-88 µm) SOIL PARTICLES
125°C DRY HEAT--CLEAN ROOM HOTPLATE
PROPCRTION POSITIVE. SS CUP-BOAT-TTC
(COMPOSITE-EXPTS. OR3310A-3338B)
FRACTION OF PARTICLES WITH VIABLE MICROORGANISMS
(PROPORTION POSITIVE)
1.0
.9
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
10
20
30
40
50
60
70
HEATING TIME IN MINUTES
NO GROWTH ON PARTICLES
HEATED FOR 80MINUTES OR
FOR 2, 3, 4, 5, OR 6 HOURS

to similar treatment in a relatively higher moisture situation. This study was of interest because earlier work with cleaned spore suspensions had suggested that dry atmospheres enhanced the thermal destruction rates of some spore strains. Therefore, it appeared useful to determine whether or not similar effects would be observed with the indigenous microflora of soil particles.

The results of the experiments carried out in dry box and clean room atmospheres are shown in Tables 9 and 10. The greatest contrast between the two environments was observed in the 110°C series (see Table 9). Of 74 particles tested at 110°C in the dry box, 9.4% were found viable after 4 hours of heat treatment. A greater reduction in viable numbers occurred among the 74 particles treated for 4 hours in the clean room where only 4% of the particles remained viable.

Dry heat treatment at 110°C in the clean room atmosphere appeared to be more effective for inactivation of soil particles. None of the particles tested in the clean room were found viable after 8 hours of heating time. By contrast, 10% of the 74 particles treated in the dry box atmosphere were still viable at the end of eight hours heating time.

Results of dry box vs clean room studies at 125°C tend to show trends similar to those observed for 110°C. Table 10 shows that in the 30 minutes heating series at 125°C, a slightly greater number of particles remained viable after heat treatment in the dry box than was found among particles heated in the clean room. Treatment of soil particles at 125°C for one hour in the clean room also resulted in a greater reduction in viable numbers than was observed in the dry box data.

The results obtained at 125°C do not show as marked a contrast as was noted at 110°C. It appears that conditioning and treatment at low moisture had either no effect or may have promoted a slight increase in resistance of the particle microflora to heat. Thus, the effect observed with soil particles seems to be different from that previously reported for studies with cleaned spores. With WAKMF soil particles, the dry box treatment had little added effect over clean room results or imparted increased protection for soil microflora. In contrast, the earlier studies with cleaned spores indicated that dry box treatment often enhanced destruction rates of spores.

Table 9

Results of Viability Tests on WAKMF Soil Particles (74-88 μm)
After 110°C Dry Heat Treatment for 4 and 8 Hours in Clean Room or Dry Box

Heating Times	Boat Number	110°C Series Proportion Positive			
		Dry Box (100 ppm)*		Clean Room (13,000 ppm)*	
		Fraction	Decimal	Fraction	Decimal
4 Hour Series (OR3347A)	1	3/37	0.081	1/37	0.027
	2	4/37	0.108	2/37	0.054
	Total	7/74	0.094	3/74	0.040
8 Hour Series (OR3347B)	1	4/37	0.108	0/37	0.000
	2	4/37	0.108	0/37	0.000
	Total	8/74	0.108	0/74	0.000

* Refers to moisture level in atmosphere of test environment.

Table 10

Results of Viability Tests on WAKMF Soil Particles (74-88 μm)
After 125°C Dry Heat Treatment for 30 and 60 Min. in Clean Room or Dry Box

Heating Times	Boat Number	125°C Series Proportion Positive			
		Dry Box (100 ppm)*		Clean Room (13,000 ppm)*	
		Fraction	Decimal	Fraction	Decimal
30 Minute Series (OR3340A)	1	7/37	0.189	6/37	0.162
	2	8/37	0.216	6/37	0.162
	Total	15/74	0.203	12/74	0.162
60 Minute Series (OR3340B)	1	5/37	0.135	4/37	0.108
	2	6/37	0.162	4/37	0.108
	Total	11/74	0.149	8/74	0.108

*Refers to moisture level in atmosphere of test environment.

CONCLUSIONS

The experimental data obtained during the studies of dry heat effects on the viability of soil particles suggest the following conclusions:

1. The use of the aluminum boat-TDT cup-TSA solid media system facilitates the assay procedure for particle viability.

2. Modifications of TSA media by addition of triphenyltetrazolium chloride improved the visual detection of microbial growth from soil particles in TDT cups.

3. Preliminary aerobic, mesophilic plate counts yielded mean values of 16.2 organisms and 2.6 organisms respectively per dark and light particles of WAKMF soil. Treatment at 110°C for one hour reduced the average counts to approximately two organisms per dark particle and one organism per 35 light particles.

4. Dry heat at 110°C caused a progressive reduction in the number of soil particles that retained viable microorganisms. Some particles remained viable through at least eight hours or more of heat treatment. After 16 hours of heating none of the particles demonstrated viability under test conditions.

5. Treatment of soil particles at 125°C caused a much more rapid loss of viable numbers than was observed at 110°C. All particles heated for 80 min. or longer appeared to be non-viable under test conditions.

6. Conditioning and heat treatment of soil particles under dry atmosphere conditions did not enhance the inactivation rates over results obtained in clear room studies. In fact, particles treated under dry box conditions showed a lower proportion of inactivation.

FUTURE WORK

1. Replicate studies to confirm the reproducibility of initial data obtained for particle viability vs time and temperature. This would involve additional heat treatment experiments with larger samples of 74-88 μm soil particles at 110°C and selected times.

2. Dry heat treatment experiments at 110°C to determine the proportion of soil particles that remain viable following selected heating times and incubation under anaerobic, mesophilic conditions.

3. Experiments to determine the proportion of particles that remain viable following 110°C heat treatment and incubation in aerobic, psychrophilic conditions.

4. Dry heat treatment and incubation of soil particles in an anaerobic, psychrophilic environment to test viability. These studies will require use of Brewer jars and perhaps some studies with Viking recovery media would also be possible in these experiments.

5. Analyses of Cape Kennedy soil particles, from size ranges available, to determine the mean microbial plate counts per particle and the mean number of heat resistant microorganisms associated with these particles.

6. Studies to determine particle viability vs time and temperature, using particles that are in the next size range smaller and larger than 74-88 μm.

7. Replication of experiments to verify the data obtained in clean room vs dry box work of earlier studies. It would be useful to determine the effect of an additional intermediate moisture level, at least for the 74-88 μm soil particles treated at 110°C.